PCT / SE 2004 / 0 0 1 0 4 9

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(71) *Sökande* *Affibody AB, Bromma SE*
Applicant (s)

(21) *Patentansökningsnummer* *0301987-4*
Patent application number

(86) *Ingivningsdatum* *2003-07-04*
Date of filing

Stockholm, 2004-07-07

För Patent- och registreringsverket
For the Patent- and Registration Office

Gunilla Larsson

Avgift
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PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

AWAPATENT AB Affibody AB
Kontor/Handläggare Ansökningsnr Vår referens
Stockholm/Niklas Mattsson/NMN SE-21004483

NEW POLYPEPTIDE

Field of the invention

The present invention is related to a new polypeptide, which binds to Human Epidermal Growth Factor Receptor 2 (in the following referred to as HER2). The polypeptide is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having at least one substitution mutation. The present invention also relates to use of such a HER2 binding polypeptide as a medicament, more particularly use thereof for the preparation of a medicament for treatment of forms of cancer characterized by overexpression of HER2.

Background

Affibody® molecules

Molecules related to protein Z, derived from domain B of staphylococcal protein A (SPA) (Nilsson B *et al* (1987) Protein Engineering 1, 107-133), have been selected from a library of randomized such molecules using different interaction targets (see e g WO95/19374; WO00/63243; Nord K *et al* (1995) Prot Eng 8:601-608; Nord K *et al* (1997) Nature Biotechnology 15, 772-777). Different target molecules have been used to select such protein Z derivatives, e g as described in Nord K *et al* (1997, *supra*). The experiments described in this reference outline principles of the general technology of selecting protein Z derivatives against given targets, rather than being a study directed towards the express objective of obtaining a molecule with high enough affinity for use in a specific therapeutic or biotechnological application.

HER2 and its role in cancer diseases

The HER2 proto-oncogene encodes the production of a 185 kD cell surface receptor protein known as the HER2 protein or receptor (Hynes NE *et al* (1994) Biochim Biophys Acta 1198:165-184). This gene is also sometimes referred to as HER2/neu or c-erbB-2. Neu was first discovered in rats that had been treated with ethylnitrosourea, and exhibited mutation of this gene (Shih C *et al* (1981) Nature 290:261-264). The mutated version of neu results in the production of a constitutively active form of the receptor, and constitutes a potent oncogene that can transform cells at low copy number (Hynes NE et *al*, *supra*).

Normal cells express a small amount of HER2 protein on their plasma membranes in a tissue-specific pattern. No known ligand to HER2 has been elucidated; however, HER2 has been shown to form heterodimers with HER1 (the epidermal growth factor receptor, EGFR), HER3 and HER4 in complex with the ligands for these receptors. Such formation of heterodimer leads to the activated HER2 receptor transmitting growth signals from outside the cell to the nucleus, thus controlling aspects of normal cell growth and division (Sundaresan S *et al* (1999) Curr Oncol Rep 1:16-22).

In tumor cells, errors in the DNA replication system may result in the existence of multiple copies of a gene on a single chromosome, which is a phenomenon known as gene amplification. Amplification of the HER2 gene leads to an increased transcription of this gene. This elevates HER2 mRNA levels and increases the concomitant synthesis of HER2 protein, which results in HER2 protein overexpression on the surface of these tumor cells. This overexpression can result in HER2 protein levels that are 10- to 100-fold greater than those found in the adjacent normal cells. This, in turn, results in increased cell division and a concomitantly higher rate of cell growth. Amplification of the HER2 gene is implicated in transforma-

tion of normal cells to the cancer phenotype (Hynes NE et *al*, *supra*; Sundaresan S et *al*, *supra*).

Overexpression of HER2 protein is thought to result in the formation of homodimers of HER2, which in turn results in a constitutively active receptor (Sliwkowski MX et *al* (1999) Semin Oncol 26(4 Suppl 12):60-70). Under these conditions, growth-promoting signals may be continuously transmitted into the cells in the absence of ligands. Consequently, multiple intracellular signal transduction pathways become activated, resulting in unregulated cell growth and, in some instances, oncogenic transformation (Hynes NE et *al*, *supra*). Thus, the signal transduction mechanisms mediated by growth factor receptors are important targets for inhibiting cell replication and tumor growth.

Breast cancer is the most common malignancy among women in the United States, with 192200 new cases projected to have occurred in 2001 (Greenlee R *et al* (2001) CA Cancer J Clin 51:15-36). In approximately 25 % of all breast cancer patients, there is an overexpression of the HER2 gene due to amplification thereof (Slamon DJ et *al* (1989) Science 244:707-712). This overexpression of HER2 protein correlates with several negative prognostic variables, including estrogen receptor-negative status, high S-phase fraction, positive nodal status, mutated p53, and high nuclear grade (Sjogren S et *al* (1998) J Clin Oncol 16(2):462-469). According to Slamon et *al* (*supra*), the amplification of the HER2 gene was found to correlate strongly with shortened disease-free survival and shortened overall survival of node-positive patients.

For these reasons, it has been, and is still, an important goal to further pursue investigations into the role of HER2 in the pathogenesis and treatment of breast cancer. The identification of molecules that interact with HER2 forms one part of this effort.

Preclinical *in vitro* studies have examined whether inhibition of HER2 activity could affect tumor cell

growth. Treatment of SK-BR-3 breast cancer cells overexpressing HER2 protein with 4D5, one of several murine anti-HER2 monoclonal antibodies, did indeed inhibit tumor cell proliferation, compared to treatment with a control monoclonal antibody. Administration of 4D5 to mice bearing human breast and ovarian cancers (xenografts) that overexpress HER2 prolonged their tumor-free survival time. Similar studies demonstrated the growth inhibition by anti-HER2 monoclonal antibodies in human gastric cancer xenografts in mice (Pietras RJ *et al* (1994) Oncogene 9:1829-1838).

Among the approaches to inhibiting the HER2 protein abundantly present on tumor cell surfaces with an antibody, one therapy has become commercially available during recent years. Thus, the monoclonal antibody trastuzumab is marketed for this purpose by F Hoffman-La Roche and Genentech under the trade name of Herceptin®.

Notwithstanding the obvious advantages shown by antibody therapy against cancers characterized by overexpression of HER2 protein, the fact remains that a variety of factors have the potential of reducing antibody efficacy (see e g Reilly RM *et al* (1995) Clin Pharmacokinet 28:126-142). These include the following: (1) limited penetration of the antibody into a large solid tumor or into vital regions such as the brain; (2) reduced extravasation of antibodies into target sites owing to decreased vascular permeability; (3) cross-reactivity and nonspecific binding of antibody to normal tissues, reducing the targeting effect; (4) heterogeneous tumor uptake resulting in untreated zones; (5) increased metabolism of injected antibodies, reducing therapeutic effects; and (6) rapid formation of HAMA and human antihuman antibodies, inactivating the therapeutic antibody.

In addition, toxic effects have been a major obstacle in the development of therapeutic antibodies for cancer (Carter P (2001) Nat Rev Cancer 1:118-129; Goldenberg DM (2002) J Nucl Med 43:693-713; Reichert JM (2002) Curr

Opin Mol Ther 4:110-118) Cross-reactivity with healthy tissues can cause substantial side effects for unconjugated (naked) antibodies, which side effects may be enhanced upon conjugation of the antibodies with toxins or radioisotopes. Immune-mediated complications include dyspnea from pulmonary toxic effects, occasional central and peripheral nervous system complications, and decreased liver and renal function. On occasion, unexpected toxic complications can be seen, such as the cardiotoxic effects associated with the HER-2 targeting antibody trastuzumab (Herceptin®) (Schneider JW *et al* (2002) Semin Oncol 29(3 suppl 11):22-28). Radioimmunotherapy with isotopic-conjugated antibodies also can cause bone marrow suppression.

Despite the recent clinical and commercial success of the currently used anticancer antibodies, a substantial number of important questions thus remain concerning the future of this therapeutic strategy. As a consequence, the continued provision of agents with a comparable affinity for HER2 remains a matter of substantial interest within the field, as well as the provision of uses of such molecules in the treatment of disease.

Disclosure of the invention

It is an object of the present invention to satisfy this interest through the provision of a polypeptide that is characterized by specific binding to HER2.

A related object of the invention is an HER2 binding polypeptide which exhibits little or no non-specific binding.

It is another object of the invention to provide an HER2 binding polypeptide that can readily be used as a moiety in a fusion polypeptide.

Another object is the provision of an HER2 binding polypeptide, which solve one or more of the known problems experienced with existing antibody reagents.

A further object of the invention is to provide an HER2 binding polypeptide, which is amenable to use in therapeutic applications.

A related object is to find new forms for the treatment, inhibition and/or targeting in the clinical setting of cancer diseases characterized by an overexpression of HER2 protein.

It is also an object to provide a molecule which can be used as a reagent for the detection of HER2 at a low detection limit.

These and other objects are met by the different aspects of the invention as claimed in the appended claims. Thus, in a first aspect, the invention provides a polypeptide, which has a binding affinity for HER2 and which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 1 to about 20 substitution mutations.

In an embodiment of the polypeptide according to this aspect of the invention, the affinity thereof for HER2 is such that the K_D value of the interaction is at most 1×10^{-6} M. In another embodiment, the affinity of the polypeptide for HER2 is such that the K_D value of the interaction is at most 1×10^{-7} M.

In another embodiment, the polypeptide according to the invention binds specifically to the extracellular domain, ECD, of the HER2 protein.

In accordance herewith, the present inventors have found that it is possible to obtain a high-affinity HER2 binding polypeptide through substitution mutagenesis of a domain from SPA, and that such a polypeptide is able to interact with HER2. The inventive polypeptide finds application as an alternative to antibodies against HER2 in diverse applications. As non-limiting examples, it will be useful in the treatment of cancers characterized by HER2 overexpression, in inhibiting cell signaling by binding to the HER2 on a cell surface, in the diagnosis

of cancer both *in vivo* and *in vitro*, in targeting of agents to cells overexpressing HER2, in histochemical methods for the detection of HER2, in methods of separation and other applications. The polypeptide according to the invention may prove useful in any method which relies on affinity for HER2 of a reagent. Thus, the polypeptide may be used as a detection reagent, a capture reagent or a separation reagent in such methods, but also as a therapeutic agent in its own right or as a means for targeting other therapeutic agents to the HER2 protein. Methods that employ the polypeptide according to the invention *in vitro* may be performed in different formats, such as in microtiter plates, in protein arrays, on biosensor surfaces, on tissue sections, and so on. Different modifications of, and/or additions to, the polypeptide according to the invention may be performed in order to tailor the polypeptide to the specific use intended, without departing from the scope of the present invention. Such modifications and additions are described in more detail below, and may comprise additional amino acids comprised in the same polypeptide chain, or labels and/or therapeutic agents that are chemically conjugated or otherwise bound to the polypeptide according to the invention.

As stated above, the sequence of the polypeptide according to the present invention is related to the SPA domain sequence in that from 1 to about 20 amino acid residues of said SPA domain have been substituted for other amino acid residues. However, the substitution mutations introduced should not affect the basic structure of the polypeptide. That is, the overall fold of the C_α backbone of the polypeptide of the invention will be essentially the same as that of the SPA domain to which it is related, e g having the same elements of secondary structure in the same order etc. Thus, polypeptides fall under the definition of having the same fold as the SPA domain if basic structural properties are shared, those

properties e g resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant. This requirement of essentially conserving the basic structure of the SPA domain, upon mutation thereof, places restrictions on what positions of the domain may be subject to substitution. When starting from the known structure of the Z protein, for example, it is preferred that amino acid residues located on the surface of the Z protein may be substituted, whereas amino acid residues buried within the core of the Z protein "three-helix bundle" should be kept constant in order to preserve the structural properties of the molecule. The same reasoning applies to other SPA domains.

The invention also encompasses polypeptides in which the HER2 binding polypeptide described above is present as an HER2 binding domain, to which additional amino acid residues have been added at either terminal. These additional amino acid residues may play a role in the binding of HER2 by the polypeptide, but may equally well serve other purposes, related for example to one or more of the production, purification, stabilization or detection of the polypeptide. Such additional amino acid residues may comprise one or more amino acid residues added for purposes of chemical coupling. An example of this is the addition of a cysteine residue at the very first or very last position in the polypeptide chain, i e at the N or C terminus. Such additional amino acid residues may also comprise a "tag" for purification or detection of the polypeptide, such as a hexahistidyl (His_6) tag for interaction with chelating agents, or a "myc" tag or a "flag" tag for interaction with antibodies specific to the tag. The skilled person is aware of other alternativesThe "additional amino acid residues" discussed above may also constitute one or more polypeptide domain(s) with any desired function, such as the same binding function as the first, HER2-binding domain, or another bind-

ing function, or an enzymatic function, or a fluorescent function, or mixtures thereof.

Thus, the invention encompasses multimers of the polypeptide with affinity for HER2. It may be of interest, e g when using the polypeptide according to the invention for treatment of cancer or in a method of purification of HER2, to obtain even stronger binding of HER2 than is possible with one polypeptide according to the invention. In this case, the provision of a multimer, such as a dimer, trimer or tetramer, of the polypeptide may provide the necessary avidity effects. The multimer may consist of a suitable number of polypeptides according to the invention. These polypeptide domains according to the invention, forming monomers in such a multimer, may all have the same amino acid sequence, but it is equally possible that they have different amino acid sequences. The linked polypeptide "units" in a multimer according to the invention may be connected by covalent coupling using known organic chemistry methods, or expressed as one or more fusion polypeptides in a system for recombinant expression of polypeptides, or joined in any other fashion.

Additionally, "heterogenic" fusion polypeptides, in which the HER2 binding polypeptide constitutes a first domain, or first moiety, and the second and further moieties have other functions than binding HER2, are also contemplated and fall within the ambit of the present invention. The second and further moiety/moieties of the fusion polypeptide may comprise a binding domain with affinity for another target molecule than HER2. Such a binding domain may well also be related to an SPA domain through substitution mutation at from 1 to about 20 positions thereof. The result is then a fusion polypeptide having at least one HER2-binding domain and at least one domain with affinity for said other target molecule, in which both domains are related to an SPA domain. This makes it possible to create multispecific reagents that

may be used in several biotechnological applications, such as used as therapeutic agents or as capture, detection or separation reagents. The preparation of such multispecific multimers of SPA domain related polypeptides, in which at least one polypeptide domain has affinity for HER2, may be effected as described above for the multimer of several HER2 binding "units". In other alternatives, the second or further moiety or moieties may comprise an unrelated, naturally occurring or recombinant, protein (or a fragment thereof retaining the binding capability of the naturally occurring or recombinant protein) having a binding affinity for a target. An example of such a binding protein, which has an affinity for human serum albumin and may be used as fusion partner with the HER2 binding SPA domain derivative of the invention, is the albumin binding domain of streptococcal protein G (SPG) (Nygren P-Å *et al* (1988) Mol Recogn 1:69-74). A fusion polypeptide between the HER2 binding SPA domain-related polypeptide and the albumin binding domain of SPG thus falls within the scope of the present invention. When the polypeptide according to the invention is administered to a human subject as a therapeutic agent or as a targeting agent, the fusion thereof to a moiety which binds serum albumin may prove beneficial, in that the half-life *in vivo* of such a fusion protein may likely prove to be prolonged as compared to the half-life of the SPA domain related HER2 binding moiety in isolation (this principle has been described e g in WO91/01743).

Other possibilities for the creation of fusion polypeptides are also contemplated. Thus, the HER2 binding SPA domain-related polypeptide according to the first aspect of the invention may be covalently coupled to a second or further moiety or moieties, which in addition to, or instead of, target binding exhibit other functions. One example is a fusion between one or more HER2 binding polypeptide(s) and an enzymatically active polypeptide serving as a reporter or effector moiety. Examples of re-

porter enzymes, which may be coupled to the HER2 binding polypeptide to form a fusion protein, are known to the skilled person and include enzymes such as β-galactosidase, alkaline phosphatase, horseradish peroxidase, carboxypeptidase. Other options for the second and further moiety or moieties of a fusion polypeptide according to the invention include fluorescent polypeptides, such as green fluorescent protein, red fluorescent protein, luciferase and variants thereof.

Other options for the second and further moiety or moieties of a fusion polypeptide according to the invention include a moiety or moieties for therapeutic applications. In therapeutic applications, other molecules may also be conjugated, covalently or non-covalently, to the inventive polypeptide by other means. Non-limiting examples include enzymes for "ADEPT" (antibody-directed enzyme prodrug therapy) applications using the polypeptide according to the invention for direction of the effector enzyme (e g carboxypeptidase); proteins for recruitment of effector cells and other components of the immune system; cytokines, such as IL-2, IL-12, TNFα, IP-10; procoagulant factors, such as tissue factor, von Willebrand factor; toxins, such as ricin A, *Pseudomonas* exotoxin, calcheamicin, maytansinoid; toxic small molecules, such as auristatin analogs, doxorubicin. Also, the above named additional amino acids (notably hexahistidine tag, cysteine), provided with the aim of conjugating chelators for radioisotopes to the polypeptide sequence, are contemplated, in order to easily incorporate radiating nuclides for diagnosis (e g In^{111}, I^{125}, Tc^{99}) or therapy (e g Y^{90}, I^{131}, At^{211}).

The invention encompasses polypeptides in which the HER2 binding polypeptide described above has been provided with a label group, such as at least one fluorophore, biotin or a radioactive isotope, for example for purposes of detection of the polypeptide.

With regard to the description above of fusion proteins incorporating the HER2 binding polypeptide according to the invention, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between the HER2 binding moiety or moieties on the one hand, and moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein. Thus, for example, said first moiety may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein.

An example of an SPA domain for use as a starting point for the creation of a polypeptide according to the invention is protein Z, derived from domain B of staphylococcal protein A. As pointed out in the Background section, this protein has previously been used as a scaffold structure for the creation of molecules, denoted Affibody® molecules, capable of binding to a variety of targets. The 58 amino acid sequence of unmodified protein Z, denoted Z_{wt}, is set out in SEQ ID NO:1 and illustrated in Figure 1.

In an embodiment of the polypeptide according to the invention, it is related to a domain of SPA in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 4 to about 20 substitution mutations. Other embodiments may have from 1 to about 13 substitution mutations, or from 4 to about 13 substitution mutations.

In a more specific embodiment of the polypeptide according to the invention, its sequence corresponds to the sequence set forth in SEQ ID NO:1 having from 1 to about 20 substitution mutations, such as from 4 to about 20, from 1 to about 13 or from 4 to about 13 substitution mutations.

The polypeptide according to the invention may in some embodiments correspond to the sequence set forth in

SEQ ID NO:1, which sequence comprises substitution mutations at one or more of the positions 13, 14, 28, 32 and 35. Additionally, the sequence of the polypeptide according to the invention may comprise substitution mutations at one or more of the positions 9, 10, 11, 17, 18, 24, 25 and 27.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 13 from phenylalanine to tyrosine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 14 from tyrosine to tryptophan.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 28 from asparagine to arginine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 32 from glutamine to arginine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 35 from lysine to tyrosine.

A preferred polypeptide according to the invention corresponds to SEQ ID NO:1, comprising at least the following mutations: F13Y, Y14W, N28R, Q32R and K35Y.

Examples of specific sequences of different embodiments of the polypeptide according to the invention, each comprising one or more of the specific mutations described above, are set out in SEQ ID NO:2-5 and illustrated in Figure 1.

As an alternative to using the unmodified SPA domain, the SPA domain may also be subjected to mutagenesis in order to increase the stability thereof in alkaline

conditions. Such stabilization involves the site-directed substitution of any asparagine residues appearing in the unmodified sequence with amino acid residues that are less sensitive to alkaline conditions. When using the polypeptide according to the invention as an affinity ligand in affinity chromatography, this property of having a reduced sensitivity to alkali provides benefits; affinity chromatography columns are frequently subjected to harsh alkali treatment for cleaning in place (CIP) between separation runs, and the ability to withstand such treatment prolongs the useful lifetime of the affinity chromatography matrix. As an example, making use of protein Z as starting point, the polypeptide according to the invention may, in addition to the substitution mutations conferring HER2 binding, have modifications in that at least one asparagine residue selected from N3, N6, N11, N21, N23, N28, N43 and N52 has been substituted with an amino acid residue that is less sensitive to alkaline treatment. Non-limiting examples of such polypeptides are those having the following sets of mutations (with respect to the sequence of Z_{wt}): N3A; N6D; N3A, N6D and N23T; N3A, N6D, N23T and N28A; N23T; N23T and N43E; N28A; N6A; N11S; N11S and N23T; N6A and N23T. Thus, these SPA domains, as well as other SPA domains that have been subjected to asparagine mutation for stability reasons, may all be subjected to further substitution mutation of amino acid residues in order to obtain the HER2 binding polypeptide of the invention. Alternatively, an HER2 binding polypeptide of the invention which comprises asparagine residues may be subjected to further mutation to replace such residues. Evidently, this latter alternative is only possible to the extent that the HER2 binding capability of such a molecule is retained.

Another aspect of the present invention relates to a nucleic acid molecule comprising a sequence which encodes a polypeptide according to the invention.

A further aspect of the present invention relates to an expression vector comprising the nucleic acid molecule of the previous aspect, and other nucleic acid elements that enable production of the polypeptide according to the invention through expression of the nucleic acid molecule.

Yet another aspect of the present invention relates to a host cell comprising the expression vector of the previous aspect.

The latter three aspects of the invention are tools for the production of a polypeptide according to the invention, and the skilled person will be able to obtain them and put them into practical use without undue burden, given the information herein concerning the polypeptide that is to be expressed and given the current level of skill in the art of recombinant expression of proteins. As an example, a plasmid for the expression of unmodified protein Z (see e g Nilsson B *et al* (1987), *supra*) may be used as starting material. The desired substitution mutations may be introduced into this plasmid, using known techniques, to obtain an expression vector in accordance with the invention.

However, the polypeptide according to the invention may also be produced by other known means, including chemical synthesis or expression in different prokaryotic or eukaryotic hosts, including plants and transgenic animals.

The present invention also concerns different aspects of using the above-described HER2 binding polypeptide, as well as various methods for treatment, diagnosis and detection in which the polypeptide is useful due to its binding characteristics. When referring to the "HER2 binding polypeptide" in the following description of these uses and methods, this term is intended to encompass the HER2 binding polypeptide alone, but also all those molecules based on this polypeptide described above that e g incorporate the HER2 binding polypeptide as a

moiety in a fusion protein and/or are conjugated to a label or therapeutic agent and/or are provided with additional amino acid residues as a tag or for other purposes. As explained above, such fusion proteins, derivatives etc form a part of the present invention.

Thus, in one such aspect, the invention provides use of the HER2 binding polypeptide as described herein as a medicament.

In a further aspect, the invention provides use of the HER2 binding polypeptide as described herein in the preparation of a medicament for the treatment of at least one form of cancer characterized by overexpression of HER2. One particular form of cancer characterized by overexpression of HER2 is a breast cancer. As described in the Background section, approximately 25 % of all breast cancer patients show an overexpression of HER2 (Slamon DJ *et al, supra*).

Without wishing to be bound by this theory, the polypeptide described herein is thought to be useful as a therapeutic agent based on at least one of the following mechanisms: (i) Potentiation of chemotherapy (cytotoxic), in that administration of the polypeptide will function in synergy with existing and coming chemotherapies and hormonal therapies. Blocking of the HER2 protein on cell surfaces has been shown to prevent DNA repair following the impact of DNA-damaging drugs (Pietras RJ *et al* (1994) Oncogene 9:1829-1838). (ii) Inhibition of the proliferation of tumor cells (cytostatic). This reasoning is based on the observation that downregulation of HER2 protein occurs when a molecule (antibody) attaches to the HER2 protein on the cell surface, causing some receptors to be endocytosed, limiting the signal for further cell growth (Baselga J *et al* (1998) Cancer Res 58:2825-2831; Sliwkowski MX *et al, supra*).

A related aspect of the present invention is the provision of a method for the treatment of at least one form of cancer characterized by overexpression of HER2,

which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a composition, which comprises a HER2 binding polypeptide as described herein as an active substance.

The HER2 binding properties of the polypeptide according to the invention, together with the suitability of the polypeptide for the creation of fusion proteins and/or labeled binding molecules, means that the polypeptide may also be useful for targeting of other active substances to the site of a tumor which comprises cells that overexpress HER2. Thus, another aspect of the present invention is the provision of use of the HER2 binding polypeptide as described herein in conjugated to an substance with anti-cancer activity for delivery of said substance to cells that overexpress HER2. The conjugated substance may also be one that functions to elicit a response of the subject's endogenous immune system. Natural killer (NK) cells, or other effectors of the immune system, may be attracted to the complex of HER2 and HER2 binding polypeptide on the cell's surface through the provision of a fusion moiety that serves the function of recruiting such effectors. The NK cells or other effectors, having detected that the cell is abnormal, attach to the HER2 binding fusion protein. Eventually, the cancer cell is consumed by the NK cells (Sliwkowski MX et al, supra; Pegram MD et al (1997) Proc Am Assoc Cancer Res 38:602, Abstract 4044).

Such an active substance may be a protein coupled to the HER2 binding polypeptide by fusion or by chemical linkage, such as chosen among effector enzymes for "ADEPT" (antibody-directed enzyme prodrug therapy) applications; proteins for recruitment of effector cells and other components of the immune system; cytokines, such as IL-2, IL-12, TNFα, IP-10; procoagulant factors, such as tissue factor, von Willebrand factor; toxins, such as ricin A, *Pseudomonas* endotoxin, calcheamicin, maytansinoid. Alternatively, the active substance may be a cyto-

toxic drug, such as auristatin analogs or doxorubicin, or a radioactive isotope (e g Y^{90}, I^{131}, At^{211}), which isotope may be associated with the HER2 binding polypeptide directly, or associated via a chelating agent, such as the well known chelators DOTA or DTPA.

In a related aspect, the invention also provides a method of directing a substance having an anti-cancer activity to cells overexpressing HER2 *in vivo*, comprising administering a conjugate of said active substance and a HER2 binding polypeptide as described herein to a patient. The conjugate is suitably as described in the preceding paragraph.

Another aspect of the present invention is the use of the HER2 binding polypeptide as described herein for the detection of HER2 in a sample. For example, such detection may be performed with the aim of diagnosing disease states characterized by overexpression of HER2. The detection of HER2 presence in a sample may be performed *in vitro* or *in vivo*. The sample in question may e g be a biological fluid sample or a tissue sample. A common method, in use today with antibodies directed against HER2, which method may be adapted for use with the HER2 binding polypeptide of the present invention, is histochemical detection of HER2 presence used for identification of HER2 protein overexpression in fresh, frozen, or formalin-fixed, paraffin-embedded tissue samples. For the purposes of HER2 detection, the polypeptide according to the invention may again be used as part of a fusion protein, in which the other domain is a reporter enzyme or fluorescent enzyme. Alternatively, it may be labeled with one or more fluorescent agent(s) and/or radioactive isotope(s), optionally via a chelator. Suitable radioactive isotopes include In^{111}, I^{125} and Tc^{99}.

Yet another aspect of the present invention is constituted by the use of an HER2 binding polypeptide as described herein in a method of detecting HER2 in a biological fluid sample. This method comprises the steps of

(i) providing a biological fluid sample from a patient to be tested, (ii) applying an HER2 binding polypeptide as described herein to the sample under conditions such that binding of the polypeptide to any HER2 present in the sample is enabled, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide. The amount of the detected bound polypeptide is correlated to the amount of HER2 present in the sample. In step (ii), the application of HER2 binding polypeptide to the sample may be performed in any suitable format, and includes for example the situation when the HER2 binding polypeptide is immobilized on a solid support with which the sample is brought into contact, as well as set-ups in which the HER2 binding polypeptide is present in solution.

Another, related, aspect of the present invention is a method for the detection of HER2 in a sample, comprising the steps of (i) providing a tissue sample suspected of containing HER2, for example a cryostat section or a paraffin-embedded section of tissue, (ii) applying an HER2 binding polypeptide according to the invention to said sample under conditions conducive for binding of the polypeptide to any HER2 present in the sample, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide. The amount of the detected bound polypeptide is correlated to the amount of HER2 present in the sample.

Also provided by the present invention is a kit for diagnosis of HER2 overexpression in a tissue sample, comprising the HER2 binding polypeptide according to the invention fused to a reporter enzyme (such as alkaline phosphatase or horseradish peroxidase), reagents for detection of enzyme activity, and positive and negative control tissue slides.

Also provided by the present invention is a kit for diagnosis of HER2 overexpression in a tissue sample, comprising the HER2 binding polypeptide according to the invention fused to a tag for detection by an antibody (such

as a flag tag or myc tag), a primary antibody specific for the tag, a secondary antibody specific for the primary antibody and conjugated to a reporter enzyme, reagents for detection of enzyme activity, and positive and negative control tissue slides.

One area in diagnosis applications is the *in vivo* detection of cancer lesions. The invention provides a kit for performing such diagnosis, which kit comprises the HER2 binding polypeptide according to the invention labeled with a chelator, a diagnostic radioactive isotope (non-limiting examples are In^{111}, Tc^{99} and I^{125}), and reagents for the analysis of the incorporation efficiency.

As described above, the invention encompasses use of the HER2 binding polypeptide according to the invention to target active substances to cells that overexpress HER2, such as certain types of cancer cells. The invention also provides a kit for this purpose, comprising HER2 binding polypeptide according to the invention labeled with a chelator, a therapeutic radioactive isotope (non-limiting examples are Y^{90}, I^{131}, At^{211}), and reagents for the analysis of the incorporation efficiency.

Brief description of the drawings

Figure 1 shows an alignment of the sequences of the sequence listing. The amino acid positions that have been subjected to modification in the polypeptides Z_{HER2} according to the invention (represented by SEQ ID NO:2-5) are indicated in bold.

Figure 2 is a schematic illustration of the amino acid sequence of a fusion polypeptide produced in the Example. Z_{HER2} represents an antigen binding domain with a sequence selected from SEQ ID NO:2-3 and His_6 represents a hexahistidyl tag.

Figure 3 shows the result of gel electrophoresis of purified fusion proteins. Lane 1: His_6-$Z_{HER2\,A}$ (8.7 kDa); Lane 2: His_6-$Z_{HER2\,B}$ (8.7 kDa); M: Molecular weight marker (LMW-SDS Marker Kit, Amersham Biosciences #17-0446-01).

Figure 4 shows Biacore sensorgrams obtained after injection of 10 µM of the His_6-$Z_{HER2\ A}$ fusion protein over sensor chip surfaces having A: HER2, B: HIV-1 gp120, and C: BB immobilized thereto.

Figure 5 shows Biacore sensorgrams obtained after injection of 10 µM of the His_6-$Z_{HER2\ B}$ fusion protein over sensor chip surfaces having A: HER2, B: HIV-1 gp120, and C: BB immobilized thereto.

Figure 6 shows Biacore sensorgrams obtained after injection of A: 1 µM; B: 2 µM; C: 5 µM; D: 10 µM, E: 20 µM; F: 40 µM of the His_6-$Z_{HER2\ A}$ fusion protein over a sensor chip surface having HER2 immobilized thereto.

Figure 7 shows Biacore sensorgrams obtained after injection of A: 1 µM; B: 2 µM; C: 5 µM; D: 10 µM, E: 20 µM; F: 40 µM of the His_6-$Z_{HER2\ B}$ fusion protein over a sensor chip surface having HER2 immobilized thereto.

The invention will now be illustrated further through the non-limiting recital of experiments conducted in accordance therewith. In these experiments, several HER2 binding polypeptides according to the invention were selected from a library of a multitude of different SPA domain related polypeptides, and subsequently characterized.

Example

Selection and ELISA study of HER2 binding polypeptides

Library panning and clone selection

A combinatorial phage display library was prepared essentially as described in Nord K *et al* (1995, *supra*). The pool of this library which was used in the present study comprised 8.7×10^8 variants of protein Z (Affibody® molecules), with random amino acid residues at positions 9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32 and 35. Antigen binding Affibody® molecules were selected in four panning cycles using biotinylated human HER2 ex-

tracellular domain (HER2-ECD) as the target (recombinant human HER2 extracellular domain, amino acids 238-2109, provided by Fox Chase Cancer Center, Philadelphia, USA). From the outcome of the four selection cycles, 91 clones were picked for phage ELISA in order to perform an analysis of their HER2 binding activity.

Phage ELISA for analysis of HER2 binding

Phages from the clones obtained after four rounds of selection were produced in 96 well plates, and an Enzyme Linked ImmunoSorbent Assay (ELISA) was used for screening for phages expressing HER2 binding Affibody® molecules. Single colonies were used to inoculate 250 µl TSB medium (30.0 g Tryptic Soy Broth (Merck), water to a final volume of 1 l, autoclaved) supplemented with 2 % glucose and 100 µg/ml ampicillin in a deep well 96 well plate and grown on a shaker over night at 37 °C. 5 µl overnight culture was added to 500 µl TSB+YE medium (30.0 g Tryptic Soy Broth (Merck), 5.0 g yeast extract, water to a final volume of 1 l, autoclaved) supplemented with 0.1 % glucose and 100 µg/ml ampicillin in a new plate. After growing at 37 °C for 3 h, 0.5 µl of 5 x 10^{12} pfu/ml (2.5 x 10^{9} pfu) helper phage M13K07 (New England Biolabs, #N0315S) and 100 µl TSB+YE medium were added to each well, and the plates were incubated without shaking at 37 °C for 30 minutes. 300 µl TSB+YE supplemented with IPTG, kanamycin and ampicillin were added to each well to a final concentration of 1 mM IPTG, 25 µg/ml kanamycin and 100 µg/ml ampicillin, and the plates were incubated on a shaker overnight at 30 °C. Cells were pelleted by centrifugation at 2500 *g* for 15 minutes and supernatants, containing phages expressing Affibody® molecules, were used in ELISA. 100 µl of 4 µg/ml of HER2 in PBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) were added to a microtiter plate (Nunc #446612) and incubated for 1 month at 4 °C. After blocking wells with 2 % skim milk powder in PBS (blocking buffer) for 1 h at room tem-

perature, 200 µl phage-containing supernatant and 50 µl 10 % blocking buffer were added. The plates were incubated for 2 h at room temperature. A polyclonal antibody (rabbit anti-M13, Abcam #ab6188) was diluted 1:1000 in 2 % blocking buffer, and 150 µl were added to each well. The plate was incubated at room temperature for 1 h. A goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma #A-3687) was diluted 1:10000 in 2 % blocking buffer, after which 150 µl were added to each well and incubated for 1 h at room temperature. Developing solution was prepared by dissolving Sigma-104 substrate (Sigma #104-105) in a 1:1 mixture of 1 M diethanolamine, 5 mM $MgCl_2$, pH 9.8 and water (1 tablet/5 ml of 1:1 mixture). Thereafter, 180 µl of the developing solution were added to each well. Wells were washed twice with PBS-T (PBS + 0.1 % Tween-20) and once with PBS before addition of each new reagent. 25 minutes after addition of substrate, the plates were read at A_{405} in an ELISA spectrophotometer (Basic Sunrise, Tecan).

Phages encoding HER2 binders were identified using a threshold criterion of an ELISA value of A_{405} above 0.5. 48 clones gave an ELISA signal above this value, and were selected for DNA sequence analysis, together with 5 clones selected at random for which no ELISA results were available.

DNA sequence analysis

Sequencing of the DNA from the clones isolated according to the procedure above was performed with the ABI PRISM®, BigDye™ Terminator v2.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations. Plasmids were prepared and DNA encoding the Affibody® molecules was sequenced using the oligonucleotides RIT-27 (5'-GCTTCCGGCTCGTATGTTGTGTG-3') and the biotinylated NOKA-2 (5'-biotin-CGGAACCAGAGCCACCACCGG-3'). The sequences were analyzed on an ABI PRISM® 3700 Genetic Analyser (Applied Biosystems).

From the 53 clones previously selected, several clones were found to encode the same amino acid sequence. Taking these degeneracies into account, four sequences of Affibody® molecules expressed by clones selected in the ELISA binding assay are given in Figure 1 ($Z_{HER2\ A-D}$), and identified in the sequence listing as SEQ ID NO:2-5.

Cloning and protein production

Z_{HER2} polypeptides were expressed in *E. coli* cells, using expression vectors encoding constructs that are schematically illustrated in Figure 2. The polypeptides were thereby produced as fusions to an N-terminal hexahistidyl tag. The fusion polypeptides His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ were purified on Immobilized Metal ion Affinity Chromatography (IMAC) columns and analyzed on SDS-PAGE. The result of the SDS-PAGE experiment is given in Figure 3.

Biosensor analysis of fusion polypeptides

The interactions between the His-tagged Z_{HER2} variants produced according to the preceding section and HER2 were analyzed using surface plasmon resonance in a Biacore® 2000 system (Biacore AB). Human HER2, HIV-1 gp120 (Protein Sciences Corporation, #2003-MN), and BB (albumin-binding protein derived from streptococcal protein G), the latter two for use as controls, were immobilized in different flow cells by amine coupling onto the carboxylated dextran layer on surfaces of CM-5 chips, according to the manufacturer's recommendations. Immobilization of human HER2, HIV-1 gp120, and BB resulted in 1900, 6290, and 1000 resonance units (RU), respectively. A fourth flow cell surface was activated and deactivated for use as blank during injections. The His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ proteins were diluted in HBS (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P-20, pH 7.4) to a final concentration of 10 µM, and injected in random order as duplicates at a constant flow-rate of 30

µl/minute. The ability of the purified proteins His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ to interact with HER2 was confirmed, as illustrated by the sensorgrams of Figures 4 and 5, respectively.

Furthermore, kinetic studies were performed for His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$. The CM-5 chip having 1900 RU of human HER2 immobilized thereto was used. A series of six different concentrations (1 µM - 40 µM) of HER2 binding polypeptide was prepared in HBS for each of His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$, and injected in random order as duplicates at a flow-rate of 30 µl/minute. The total injection time was 50 seconds (association) followed by a wash during 6 minutes (dissociation). The surfaces were regenerated with 20 mM HCl for 10 seconds. The responses measured in reference cells (activated/deactivated surface) were subtracted from the response measured in the cells with immobilized HER2. The binding curves (sensorgrams) were analyzed using the 1:1 Langmuir binding model of the BIAevaluation 3.0.2 software (Biacore AB). As is clear from the binding curves presented in Figures 6 (His_6-$Z_{HER2\ A}$) and 7 (His_6-$Z_{HER2\ B}$), His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ both clearly bind to HER2, as evidenced by the association and dissociation curves with an indicated K_D of 10-100 nM for His_6-$Z_{HER2\ A}$ and 200-400 nM for His_6-$Z_{HER2\ B}$. Furthermore, the binding is selective, since neither of the HER2 binding polypeptides studied bind to the BB and gp120 control antigens (Figures 4 and 5).

CLAIMS

1. Polypeptide, which has a binding affinity for HER2 and which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 1 to about 20 substitution mutations.

2. Polypeptide according to claim 1, which has a binding affinity for HER2 such that the K_D value of the interaction is at most 1×10^{-6} M.

3. Polypeptide according to claim 2, which has a binding affinity for HER2 such that the K_D value of the interaction is at most 1×10^{-7} M.

4. Polypeptide according to any one of claims 1-3, the sequence of which corresponds to the sequence of SPA protein Z, as set forth in SEQ ID NO:1, comprising from 1 to about 20 substitution mutations.

5. Polypeptide according to claim 4, comprising from 4 to about 20 substitution mutations.

6. Polypeptide according to claim 4 or 5, comprising substitution mutations at one or more of the positions 13, 14, 28, 32 and 35.

7. Polypeptide according to claim 6, additionally comprising substitution mutations at one or more of the positions 9, 10, 11, 17, 18, 24, 25 and 27.

8. Polypeptide according to any one of claims 4-7, comprising a substitution mutation at position 13 from phenylalanine to tyrosine.

9. Polypeptide according to any one of claims 4-8, comprising a substitution mutation at position 14 from tyrosine to tryptophan.

10. Polypeptide according to any one of claims 4-9, comprising a substitution mutation at position 28 from asparagine to arginine.

11. Polypeptide according to any one of claims 4-10, comprising a substitution mutation at position 32 from glutamine to arginine.

12. Polypeptide according to any one of claims 4-11, comprising a substitution mutation at position 35 from lysine to tyrosine.

13. Polypeptide according to any one of claims 4-12, the amino acid sequence of corresponds to that of SEQ ID NO:1, comprising at least the following mutations: F13Y, Y14W, N28R, Q32R and K35Y.

14. Polypeptide according to any one of claims 4-13, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-5.

15. Polypeptide according to claim 14, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-3.

16. Polypeptide according to any preceding claim, in which at least one of the asparagine residues present in the domain of staphylococcal protein A (SPA) to which said polypeptide is related have been replaced with another amino acid residue.

17. Polypeptide according to claim 16, the sequence of said domain of staphylococcal protein A (SPA) corre-

sponding to the sequence of SPA protein Z as set forth in SEQ ID NO:1, and the polypeptide comprising substitution mutations at at least one position chosen from N3, N6, N11, N21, N23, N28, N43 and N52.

18. Polypeptide according to claim 17, comprising at least one of the following mutations: N3A, N6A, N6D, N11S, N23T, N28A and N43E.

19. Polypeptide according to any preceding claim, which comprises additional amino acid residues at either terminal.

20. Polypeptide according to claim 19, in which the additional amino acid residues comprise a cysteine residue at the N- or C-terminal of the polypeptide.

21. Polypeptide according to any one of claims 19-20, in which the additional amino acid residues comprise a tag, preferably chosen from a hexahistidinyl tag, a myc tag and a flag tag.

22. Polypeptide according to any one of claims 19-21, in which the additional amino acid residues comprise at least one functional polypeptide domain, so that the polypeptide is a fusion polypeptide between a first moiety, consisting of the polypeptide according to any one of claims 1-18, and at least one second and optionally further moiety or moieties.

23. Polypeptide according to claim 22, in which the second moiety consists of one or more polypeptide(s) according to any one of claims 1-18, making the polypeptide a multimer of HER2 binding polypeptides according to any one of claims 1-18, the sequences of which may be the same or different.

24. Polypeptide according to claim 22, in which the second moiety comprises at least one polypeptide domain capable of binding to a target molecule other than HER2.

25. Polypeptide according to claim 24, in which the second moiety comprises at least one polypeptide domain capable of binding to human serum albumin.

26. Polypeptide according to claim 25, in which the at least one polypeptide domain capable of binding to human serum albumin is the albumin binding domain of streptococcal protein G.

27. Polypeptide according claim 24, in which the second moiety comprises a polypeptide which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 1 to about 20 substitution mutations.

28. Polypeptide according claim 27, in which the sequence of the second moiety polypeptide corresponds to the sequence of SPA protein Z, as set forth in SEQ ID NO:1, having from 1 to about 20 substitution mutations.

29. Polypeptide according to claim 22, in which the second moiety is capable of enzymatic action.

30. Polypeptide according to claim 22, in which the second moiety is capable of fluorescent action.

31. Polypeptide according to claim 22, in which the second moiety is a phage coat protein or a fragment thereof.

32. Polypeptide according to any preceding claim, which comprises a label group.

33. Polypeptide according to claim 32, in which the label group is chosen from fluorescent labels, biotin and radioactive labels.

34. Polypeptide according to any one of the preceding claims, conjugated to a substance having an activity against cells overexpressing HER2.

35. Polypeptide according to claim 34, in which said substance having an activity against cells overexpressing HER2 is chosen from cytotoxic agents, radioactive agents, enzymes for ADEPT applications, cytokines and procoagulant factors.

36. Nucleic acid molecule comprising a sequence encoding a polypeptide according to any one of claims 1-30.

37. Expression vector comprising the nucleic acid molecule according to claim 36.

38. Host cell comprising the expression vector according to claim 37.

39. Use of a polypeptide according to any one of claims 1-35 as a medicament.

40. Use of a polypeptide according to any one of claims 1-35 in the preparation of a medicament for the treatment of at least one form of cancer characterized by overexpression of HER2.

41. Method of treatment of at least one form of cancer characterized by overexpression of HER2, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a compo-

sition, which comprises a polypeptide according to any one of claims 1-35 as an active substance.

42. Use of a polypeptide according to any one of claims 1-35 conjugated to a substance with anti-cancer activity for delivery of said substance to cells that overexpress HER2.

43. Method of directing a substance having an anti-cancer activity to cells overexpressing HER2 *in vivo*, which method comprises administering a conjugate of said substance and a polypeptide according to any one of claims 1-35 to a subject.

44. Use of a polypeptide according to any one of claims 1-35 for the detection of HER2 in a sample.

45. Method of detection of HER2 in a sample, in which method a polypeptide according to any one of claims 1-35 is used.

46. Method according to claim 45, comprising the steps: (i) providing a sample to be tested, (ii) applying a polypeptide according to any one of claims 1-35 to the sample under conditions such that binding of the polypeptide to any HER2 present in the sample is enabled, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide.

47. Method according to claim 46, in which the sample is a biological fluid sample, preferably a human blood plasma sample.

48. Method according to claim 46, in which the sample is a tissue sample, preferably a human tissue sample, more preferably a biopsy sample from a human suffering from cancer.

49. Kit for diagnosis of HER2 overexpression in a tissue sample, which kit comprises a polypeptide according to any one of claims 1-35 fused to a reporter enzyme, reagents for detection of activity of said reporter enzyme, and positive and negative control tissue slides.

50. Kit for *in vivo* diagnosis of HER2 overexpression, which kit comprises a polypeptide according to any one of claims 1-35 labeled with a chelator, a diagnostic radioactive isotope, and reagents for the analysis of the incorporation efficiency.

51. Kit for performing the method of claim 43, which kit comprises a polypeptide according to any one of claims 1-35 labeled with a chelator, a therapeutic radioactive isotope, and reagents for the analysis of the incorporation efficiency.

SEQUENCE LISTING

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20 25 30

Ser Leu Ser Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

ABSTRACT

A polypeptide is provided, which has a binding affinity for HER2 and which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 1 to about 20 substitution mutations. Nucleic acid encoding the polypeptide, as well as expression vector and host cell for expressing the nucleic acid, are also provided.

Also provided is the use of such a polypeptide as a medicament, and as a targeting agent for directing substances conjugated thereto to cells overexpressing HER2.

Methods, and kits for performing the methods, are also provided, which methods and kits rely on the binding of the polypeptide to HER2.

Polypeptide	Amino acid sequence	SEQ ID NO:
Z_{wt}	VDNKFNKEQQ NAFYEILHLP NLNEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK	1
		2
$Z_{HER2\ A}$	VDNKFNKELR QAYWEIQALP NLNWTQSRAF IRSLYDDPSQ SANLLAEAKK LNDAQAPK	3
$Z_{HER2\ B}$	VDNKFNKEPK TAYWEIVKLP NLNPEQRRAF IRSLYDDPSQ SANLLAEAKK LNDAQAPK	4
$Z_{HER2\ C}$	VDNKFNKEPR EAYWEIQRLP NLNNKQKAAF IRSLYDDPSQ SANLLAEAKK LNDAQAPK	5
$Z_{HER2\ D}$	VDNKFNKEWV QAGSEIYNLP NLNRAQMRAF IRSLSDDPSQ SANLLAEAKK LNDAQAPK	

FIGURE 1

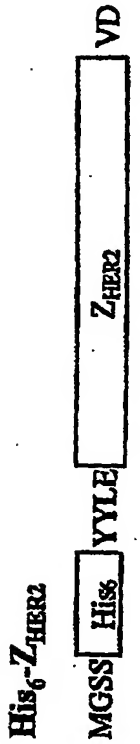

FIGURE 2

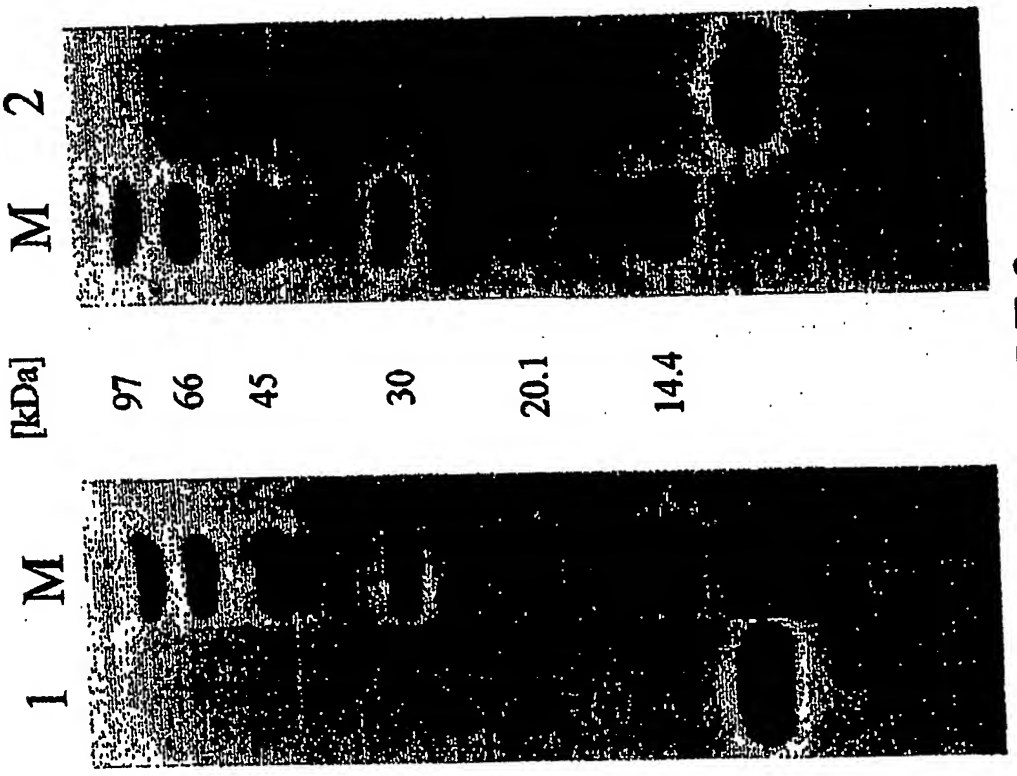

FIGURE 3

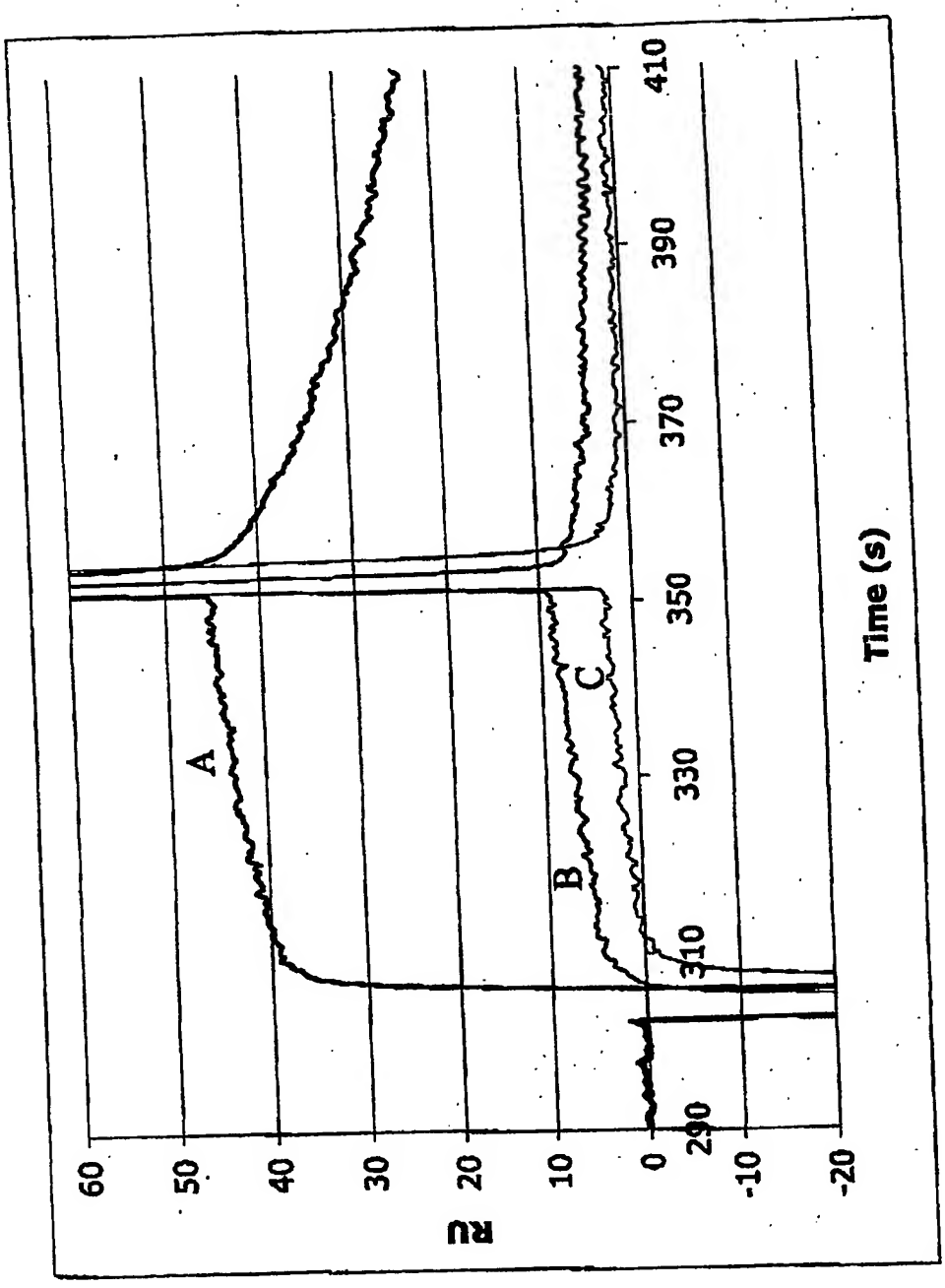

FIGURE 4

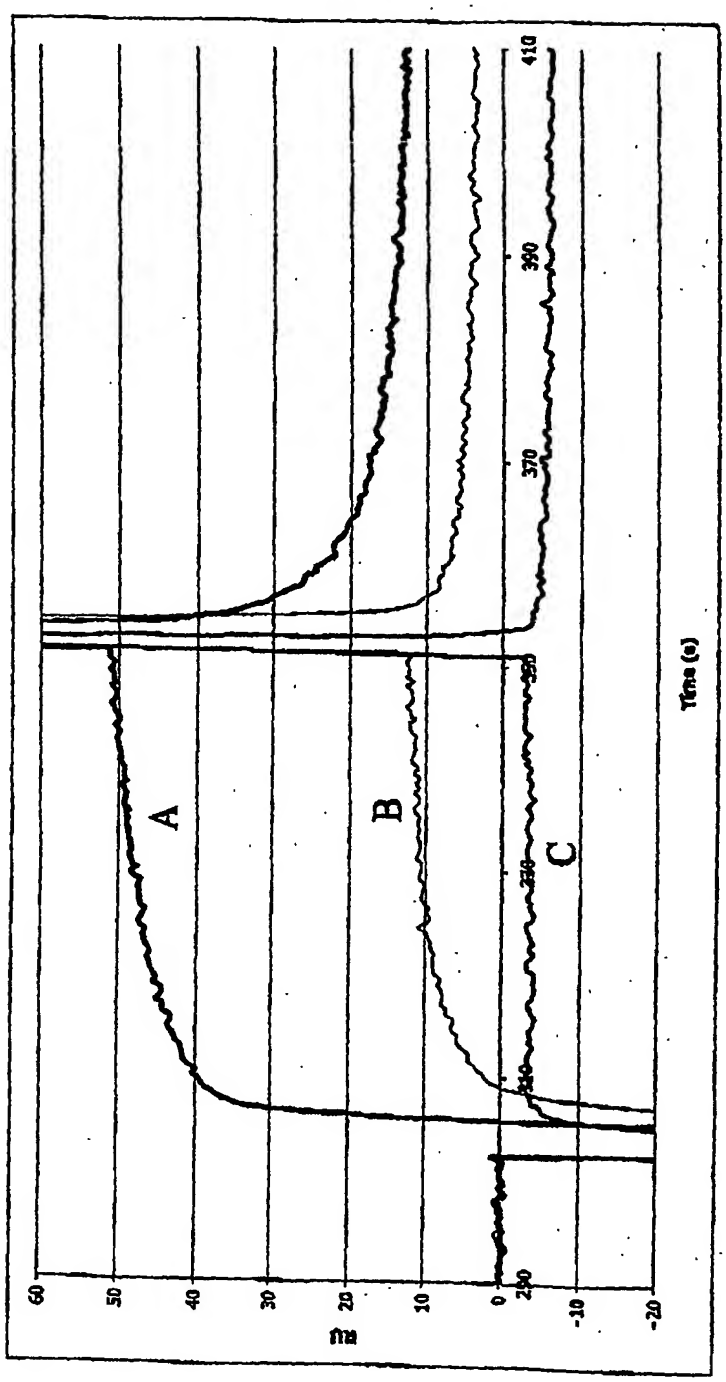

FIGURE 5

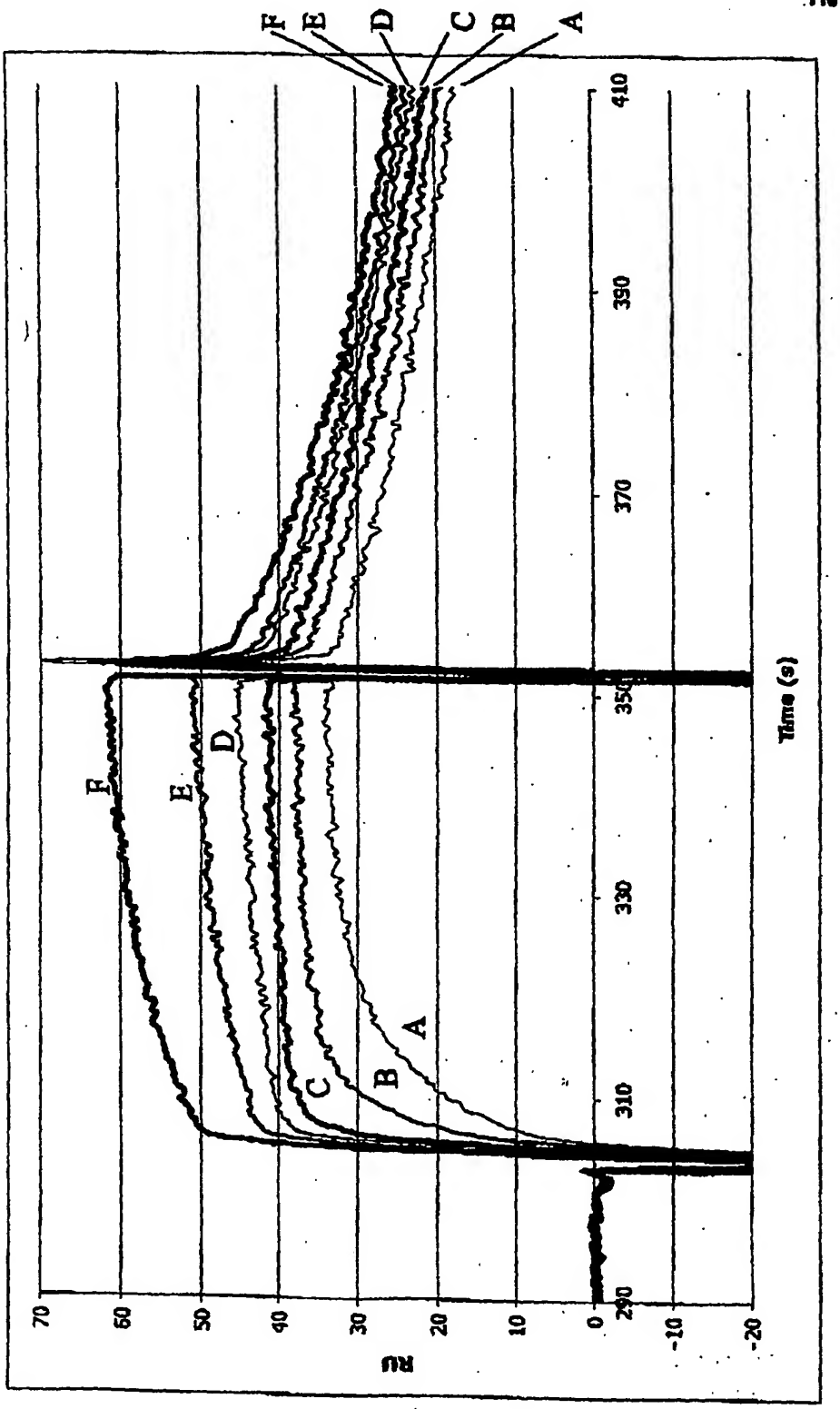

FIGURE 6

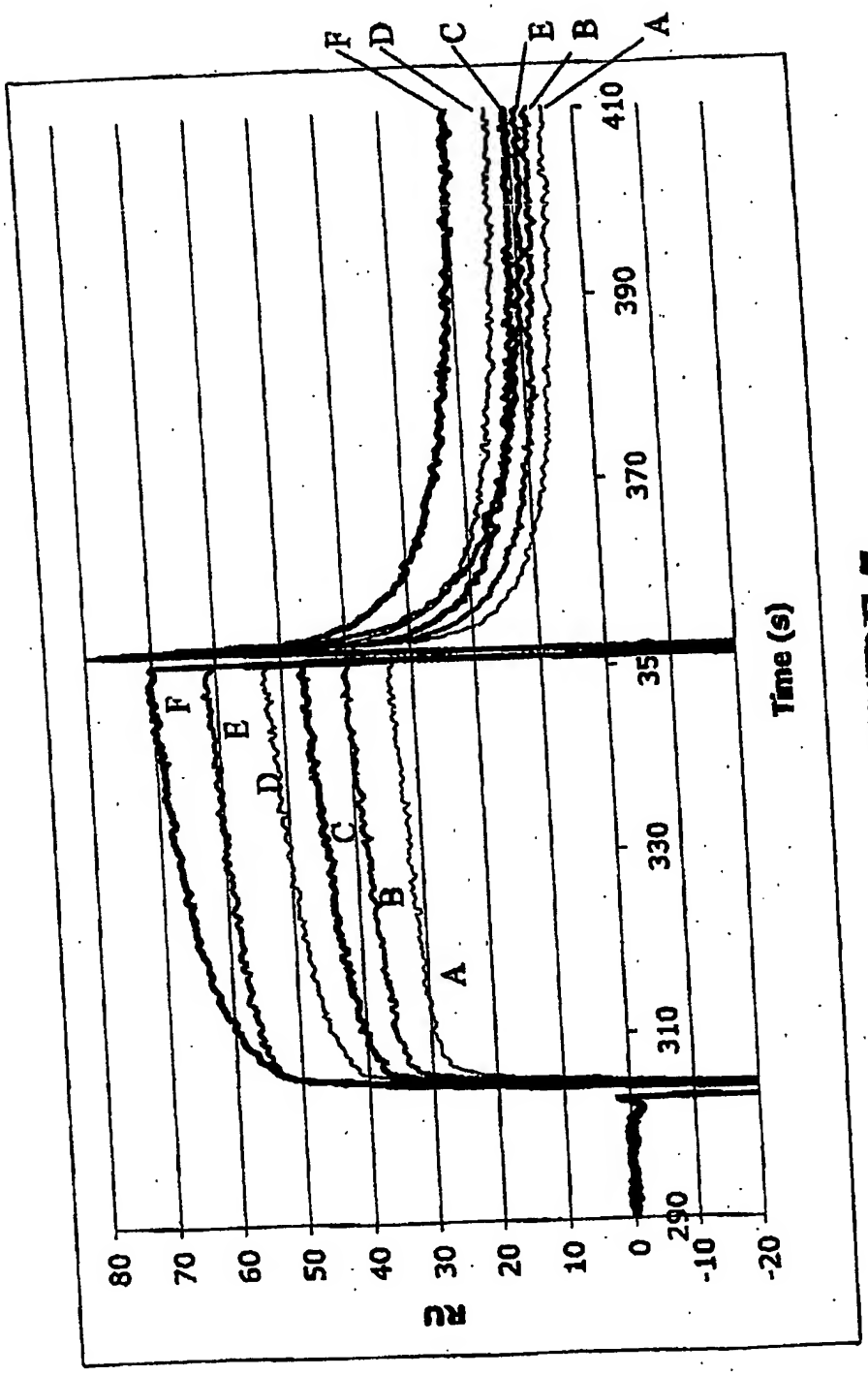

FIGURE 7

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